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Sensory cross-talk between chemotaxis behavior and the regulation of swarmer cell differentiation of Vibrio parahaemolyticus. N00014-92-J-1892

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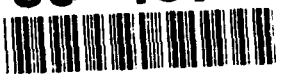

Vibrio parahaemolyticus is a major causative agent in the process known as marine biofouling, or the attachment of living organisms to submerged surfaces. V. parahaemolyticus accomplishes this attachment by the production of a differentiated hyperflagellated swarmer cell. It has been observed that mutants of V. parahaemolyticus defective in chemotaxis behavior show aberrant expression of the swarmer cell. Although chemotaxis behavior and swarmer cell differentiation are considered to be separate sensory transduction systems, such Che mutants must have a genetic defect in a protein integral to the regulation of swarmer cell differentiation, thus linking the two signal pathways together. It is the goal of this work to identify this link. We have constructed a set of Che mutants in V. parahaemolyticus and have characterized them for defects in swimming behavior. The che DNA from 3 of 10 total Che mutants was cloned into E. coli and physically characterized. Mutations in each respective cloned che gene were constructed so that allelic exchange mutagenesis could be conducted on a V. parahaemolyticus strain harboring a laf::lux transcriptional fusion.

Vibrio parahaemolyticus Swarmer cell differentiation 7
Chemotaxis flagella

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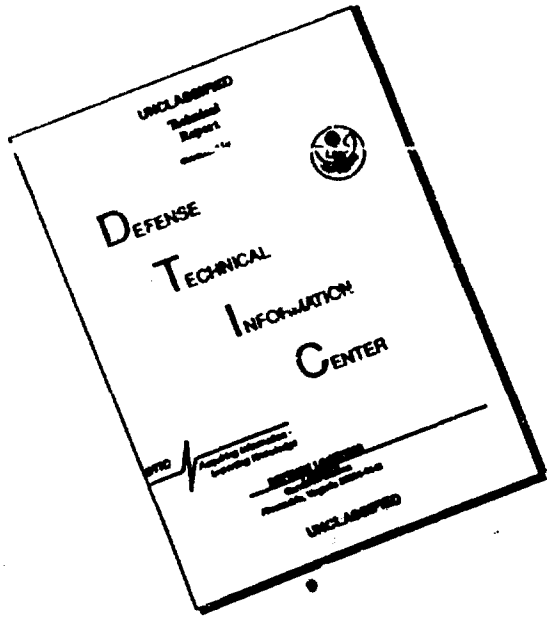

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FINAL TECHNICAL REPORT

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PRINCIPAL INVESTIGATOR: M. ROBERT BELAS

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GRANT TITLE: SENSORY CROSS-TALK BETWEEN CHEMOTAXIS BEHAVIOR AND THE REGULATION OF SWARMER CELL DIFFERENTIATION IN *VIBRIO PARAHAEMOLYTICUS*

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OBJECTIVE: It is the goal of this laboratory to understand how environmental signals control gene expression in marine bacteria. The model system we have chosen to explore is the surface-induced regulation of *Vibrio parahaemolyticus* swarmer cell differentiation. The long range goal of the proposed research is to focus on the genes and regulatory proteins they encode which control expression of *laf* (for *la*teral *f*lagella) genes. It has been observed that expression of *laf* genes is under control of factors which monitor the rotation of the polar flagellum. If the cells encounter environmental conditions which inhibit the normal flagellar rotation, a signal is sent which starts transcription of a set of 40-60 *laf* genes. This ultimately gives rise to the differentiated swarmer cell. In addition to inhibition of the rotation of the polar flagellum, it has been observed that mutations affecting normal chemotaxis behavior, but not affecting the flagellar RPM, also cause transcriptional activation of *laf* genes. The goal of the proposed research is to understand the path of signal transduction involved in the regulation of surface-induced swarmer cell differentiation, and to explain the role of cytoplasmic Che proteins in controlling the expression of *laf* genes.

ACCOMPLISHMENTS: The first aim of this project was to construct a bank of mutants in *V. parahaemolyticus* which were defective in wild-type polar flagellum motility. To accomplish this we introduced transposon mini-Mu (Tet^r, *lacZ*) into BB2203 (Fla^+ Laf^-). BB2203 was produced by EMS mutagenesis and is defective in the synthesis of lateral flagella. This strain was used to eliminate complications inherent when both the polar and lateral flagella systems are functional. Thus, BB2203 was wild-type for polar flagellum motility but defective for lateral flagella motility. Following P1 transduction of the mini-Mu transposon into *V. parahaemolyticus* and selection for tetracycline resistance, ca. 15,000 transposon-generated mutants were screened for loss of wild-type swimming motility. This screening procedure identified 54 strains defective in wild-type swimming. Defects in swimming can be characterized as one of three phenotypes. Mutants defective in the synthesis of flagellin are referred to as Fla^- mutants. Those strains which synthesize a flagellum but cannot rotate it (and thus are paralyzed) are termed Mot^-. Finally, the Che^- phenotype is used to describe those strains which can synthesize and rotate the flagellum, but which are unable to control the frequency of reversal of rotation. Since Fla^- and Mot^- phenotypes disrupt the RPM of flagellar rotation, our goal was to find Che^- mutants which by definition have a wild-type flagellar RPM, but are mutant in the

rotational bias of the flagellum. We found 10 such Che$^-$ mutants. Thus, the first goal of the project was successfully completed.

The second goal of our research was to characterize the behavior of each of the Che$^-$ mutants, and then to clone the flanking DNA from a selected group of these mutants. Che$^-$ mutants can be subdivided into two categories, those which are smooth swimmers (CCW bias of flagellar rotation) and cells which are tumbly swimmers and are biased towards predominant CW flagellar rotation. Each of the 10 strains were examined using video microscopy and designated as either a smooth or tumbly swimmer. Of the 10 original Che$^-$ strains, 4 were found to smooth swimmers, while 3 were tumbly swimmers. The other 3 strains could not be conclusively placed into either group and were not studied further. Not surprisingly, each of the total of 7 strains showed abnormal expression of *laf* genes, i.e., they produced differentiated swarmer cells under noninducing condition. This confirms earlier observations establishing a link between Che$^-$ phenotype and *laf* expression. A representative from the smooth swimming biased group (strain YL112) and from the tumbly swimming biased group (YL150) was chosen for further study.

Cloning of *che* gene DNA from each of the two mutants identified and characterized previously relied on the use of the transposon-mediated drug resistance. Our cloning strategy was to isolate chromosomal DNA from each strain, partial digest the DNA with a suitable restriction enzyme and ligate the resulting DNA fragments to a cosmid vector. Selection for the antibiotic resistance marker (from the transposon) would identify the clone as that containing a portion of the *che* gene flanking the transposon insert. We have found plasmid clones harboring che gene sequences for each of the two Che$^-$ mutants (designated as pMM112 and pMM150) described above, thus, we have fulfilled the second goal of this project.

The third aim of this research was to construct a site-specific mutation in each of the two *che* genes in *V. parahaemolyticus* BB1301 (*laf::lux* 1301). This strain contains a promoterless luciferase reporter gene cassette inserted into one of the *laf* genes actively expressed during surface induction. Thus, strain BB1301 (*laf::lux* 1301) produces light when the signal initiating *laf* transcription is active. The *che* gene fragments from pMM112 and pMM150 were used to locate the respective intact *che* gene from a cosmid library constructed from partially digested wild-type vibrio genomic DNA. Physical mapping of each che gene using restriction enzymes sites as markers has identified a unique six cutter site in each gene. Our plan was to insert a selectable marker, in this case chloramphenicol resistance, into each *che* gene while still harbored on a plasmid in *E. coli*. These constructions were then to have been moved into *V. parahaemolyticus* BB1301 (*laf::lux* 1301) where replication of the plasmid is not possible. Therefore, the plasmid would suicide resulting in the integration of the disrupted che gene into the chromosome. We have begun work to construct these strains and would have accomplished our third aim in another several weeks. Unfortunately, the untimely termination of this grant may have prevented completion of this part of the research. We are currently finalizing what can be completed in the meantime.

The last goal of the project as described in the original proposal was to measure expression of *laf* gene transcription as a consequence of the site-specific mutation in each of the two *che* genes (designated *che112* and *che150* in our study). This part of the project has yet to begin. We anticipate that we may be able to partially complete some of this work with additional salary and supply funds in the future.

Although not originally proposed, we have also started to identify the cytoplasmic transcriptional activators responsible for induction of *laf* genes as part of our effort on this ONR-sponsored grant. We strongly believe that the Che system serves to modulate the signal directed from the surface and does not pay an active regulatory role. (No Che protein has been observed to possess DNA binding ability in either *E. coli* or *S. typhimurium*.) In order for Che to modulate the surface-inducing signal, it is necessary to develop a model with at least one other central component, a DNA binding regulatory protein (or proteins) that activates or prevents transcription at each of the 50 or more *laf* genes. We have assigned the genotype of *lafR* (for ***laf***eral ***f***lagella ***R***egulatory protein) to that gene (or those genes) necessary to activate transcription. To accomplish the task of finding regulators of *laf* genes, we first had to develop a new set of transposable elements and vectors to be used in strains harboring mini-Mu-generated transcriptional fusion, e.g., strain BB1301 (*laf::lux* 1301). (The use of mini-Mu as a secondary mutagen in strains already harboring a mini-Mu transposon is not possible due to the immunity induced in the cell by the first Mu infection.) To overcome this problem, Dr. Roger Chien has developed a set of mini-Tn*5* transposons carried on a broad-host range vector. This vector may be conjugally mated into recipient *V. parahaemolyticus* using a suitable *E. coli* donor. A second incompatible plasmid is then conjugally mated into the same strain. The two plasmids cannot exist simultaneously due to their incompatible nature, thus genetic selection for the second plasmid results in the loss of the transposon-bearing plasmid. The loss of the transposon-bearing plasmid results in transposition of mini-Tn*5* to new sites on *V. parahaemolyticus* DNA. Dr. Chien has used this strategy to develop a bank of mutants in BB1301 (*laf::lux* 1301) and has screened the bank for those cells showing either constitutive light production or no light production. The rationale being that if the *laf* system is under negative control then mutation of the *lafR* loci responsible for control of *laf* transcription would result in constitutive expression of *laf*. Conversely, if the system is positively controlled, then mutation of the regulatory region(s) would prevent *laf* expression under all circumstances.

Using the strategy outlined above, Dr. Chien has found 72 mutants with abnormal regulation of light in the *laf::lux* 1301 background. As indicated earlier, these mutants fall into two broad classes. The first class were constitutive light producers indicating the *laf* expression was active at all times. This phenotype could be the result of either 1) disruption of *fla*, *mot*, or *che* genes, or 2) mutation of a *lafR* gene. The second class of mutants did not produce light at any time. Constitutive "darks", as these mutants are called, can be due to either disruption of the *lux* cassette (no luciferase) or disruption of a *lafR* gene. To eliminate unwanted *fla*, *mot*, *che*, or *lux* mutations, the 72 original mutants were screened for swimming and chemotaxis behavior, as well as for an intact *lux* cassette (in this case, through Southern blot analysis of genomic DNA with a radioactive *lux* DNA probe). From the original 72 mutants, 16 were considered to be putative mutations in a *lafR* gene. Initial analyses to confirm genetic linkage of these mutations were begun in late December 1991, but were stopped due to termination of funding. Due to visa restrictions, Dr. Chien, a native of Taiwan, left this laboratory January 31, 1992 to return to a university position in Taiwan. Mr. David Flaherty and the Principal Investigator will continue the research on regulatory proteins of *laf* gene in a limited capacity due to termination of funding.

As part of the 6 month continuation of this project, Dr. Michael Montgomery continued his research on the interaction of cytoplasmic Che proteins in the regulation of *laf* gene expression. He attempted to finalize genetic characterization of both *che112* and *che150* genes, but did not place them into vibrio as originally planned. The scope of his work has had to be reduced due to termination of our original grant. Mr. David Flaherty assisted the Principal

Investigator in completing the *lafR* analyses originally started by Dr. Chien. As with Dr. Montgomery's research, the work on *lafR* was not fully completed due to lack of funds.

Due to the short time span of this request, it was not be possible to specifically address questions concerning the nature of each of the *lafR* loci we had identified through mutagenesis. Instead, we proposed to complete the construction of a complete set of mini-Tn*5* transposon vectors for use in the mutagenesis of marine bacteria. We have constructed two such vectors and have all the starting material needed to construct six more. These would provide transposons carrying either spectinomycin, chloramphenicol, tetracycline, or kanamycin resistance. Further, transcriptional and translational fusions to either *lacZ*, *lux*, *phoA*, or *xylE* could also be incorporated into these new transposons. Since we have all the starting materials these constructions require nothing more sophisticated than restriction enzyme digests, ligations, and genetic selections and screens. We believe that construction of such a set of transposon will be highly useful to all research employing marine bacteria and hope to accomplish these constructions in the future.

SIGNIFICANCE: We have approached the question of environmental regulation of genes from a unique perspective in this grant proposal. *Vibrio parahaemolyticus* and *V. alginolyticus* are the only known marine bacteria with the ability to sense surfaces. This fact in and of itself makes the study of surface-induced swarmer cell differentiation an interesting phenomenon to study. More over, we have established from past work with *V. parahaemolyticus* that the organism senses surfaces by monitoring the rotation of the polar flagellum. The signal from that event is then somehow used to activate the transcription of a set of between 40 and 60 *laf* genes which encode the proteins which are needed to produce the swarmer cell. In the current research we have extended our line of questioning to those events in the cytoplasm of the bacterium required to transduce the surface-induction signal to sites on the chromosome that are activated during the surface-induced response. We have focused on cytoplasmic chemotaxis proteins and on those DNA binding regulatory proteins that specifically interact to either turn the system on and off, or modulate the response. This approach is the obviously and logical choice to take in a more complete understanding of the global swarmer cell differentiation response.

Our research on the relationship between chemotaxis behavior and surface-induced swarmer cell differentiation has progressed to the point where we have identified, characterized, and cloned two *che* genes from *V. parahaemolyticus.* Each gene encodes for a protein whose absence produces a constitutive swarmer cell phenotype. Thus, although the mechanism remains a mystery, the proteins encoded by each *che* gene must be involved in the processing of information during surface-induction of laf genes. Molecular characterization of these *che* genes may provide the clue to understand how the Che proteins interact with the swarmer cell differentiation control circuit. Our current cloning and DNA sequence analysis should provide a wealth of information on this subject. Furthermore, since Che proteins are not known to be involved in any other genetic control circuit in either *E. coli* or *S. typhimurium*, the information from DNA sequencing may provide information on this novel feature of *V. parahaemolyticus* Che proteins. In the future, should funding be available, we plan to finish the construction of the site-specific che mutations (*che112* and *che150)* in BB1301 (*laf::lux* 1301). These constructions will be used to measure *laf* transcription and should provide indisputable data regarding the interaction of the V. parahaemolyticus chemotaxis system and genetic control of the swarmer cell differentiation global regulatory pathway.

Our analysis of the DNA binding protein regulators of *V. parahaemolyticus* swarmer cell differentiation is still in its beginning stages. We are deeply interested in continuing the analysis of these *lafR* loci and will do so when funding becomes available. Although a byproduct of our research, we have developed a new set of transposable elements and vectors for introducing transposons in marine bacteria. We have tested these transposons in *V. parahaemolyticus*, *Pseudomonas fluorescens*, and *Proteus mirabilis* and in each case the system appears to work flawlessly. Since of the main obstacles to the further analysis of marine bacteria has been the development of mutants, our system of transposon mutagenesis should be of use to a wide audience of scientists.

Second, one of the most tantalizing pieces of data to emerge from this study is that there appears to be more than one genetic locus involved in *laf* control. The idea of multiple regulatory genes acting in a cascade is not new in prokaryotic molecular genetics, but these are the first data to show that such a cascade may be required for the control of swarmer cell differentiation. Besides the obvious significance of identifying and partially characterizing these genes and their corresponding proteins, this aspect of research is especially significant because it will likely be able to complete the sensory transduction chain in this surface-induction event. Combined with what we already know about the polar flagellum as a sensor of surfaces, and further compounded with the knowledge of the interaction of Che proteins, the information on *lafR* genes will provide a continuum to the flow of information into the cell. Although we are still not at this stage, we anticipate that when the analysis of the *lafR* genes is completed we should be able to provide an accurate picture of the chain of events (at the molecular level) that starts at the signal from the surface and ultimately ends by initiating transcription of a surface-induced laf gene. The significance of this is obvious and will most likely be the first elucidated signal transduction pathway in bacteria where the signal source is a submerged surface.

INVENTIONS: None.

PUBLICATIONS AND REPORTS:

1. Lai, K. and R. Belas. 1990. Genetic analysis of chemotaxis mutants of *Vibrio parahaemolyticus*. Abstr. 90th Ann. Meeting Amer. Soc. Microbiol. I75.

2. Montgomery, M. and R. Belas. 1992. The relationship between chemotaxis behavior and swarmer cell differentiation in *Vibrio parahaemolyticus*. Abstr. 92th Ann. Meeting Amer. Soc. Microbiol.

MANUSCRIPTS IN PREPARATION:

1. Chien, R. H., D. Flaherty, and R. Belas. Genetic analysis of transcriptional activators of *Vibrio parahaemolyticus laf* genes. To be submitted to the Journal of Bacteriology.

2. M. Montgomery and R. Belas. Mutagenesis, molecular cloning, and complementation analysis of *Vibrio parahaemolyticus che* genes. To be submitted to the Journal of Bacteriology.

3. Montgomery, M. and R. Belas. The relationship between chemotaxis behavior and swarmer cell differentiation in *Vibrio parahaemolyticus*. To be submitted to Molecular Microbiology.

PERSONNEL:	Dr. Michael Montgomery	Postdoctoral Associate	1991-present
	Dr. Roger Chien	Postdoctoral Associate	1991-1992

Mr. David Flaherty	Faculty Research Assistant	1990-present
Mr. Eric Witzel	Faculty Research Assistant	1989-1990
Mr. Kent Lai	Graduate Student	1989-1990

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